

AMYLASE

FIELD OF THE INVENTION

The present invention relates to amylases and their addition to dough to prevent staling of dough-based products.

5 BACKGROUND OF THE INVENTION

Endo-amylases are often added to dough to make the dough more soft and to improve the moistness, often in combination with an exo-amylase, e.g. a maltogenic alpha-amylase. However, the addition of endo-amylases of bacterial origin, e.g. *B. amyloliquefaciens* amylase easily gives a risk of overdosing, giving loss of elasticity and a too gummy crumb.

10 Fungal amylases are often added to dough, but they only provide little effect on the softness

A fungal amylase (Taka amylase) from *Aspergillus oryzae* is described in Pdb file 6taa (available at www.rcsb.org).

SUMMARY OF THE INVENTION

The inventors have identified an amylase in fungal strains of *Valsaria* and found that
15 the amylase can increase the shelf life of baked products. Particularly, the novel amylase in combination with an exo-amylase further improves the anti-staling effect of the exo-amylase. The improved anti-staling may be an improved the softness of bread crumb without a detrimental effect on crumb elasticity or even with an improved elasticity.

The amino acid sequence of the novel amylase was found to include a catalytic do-
20 main and a carbohydrate-binding domain (CBM), each of which can be used separately. Accordingly, the invention provides a polypeptide with a sequence including a catalytic core and a polypeptide with a sequence including a carbohydrate-binding domain (CBM).

The novel amylase is thermostable, and the inventors found that a combination of two amylases can be used for anti-staling, where one is a thermostable amylase (particularly a
25 fungal amylase) which includes both a catalytic core and a CBM, and the other is an exo-amylase.

The invention also provides a polypeptide having an amino acid sequence which can be obtained from the mature polypeptide (particularly the catalytic core of SEQ ID NO: 2 or 19 by substitution, deletion, and/or insertion of one or more amino acids and a polynucleotide hav-
30 ing a sequence that can be derived from SEQ ID NO: 1 or 18 by substitution, deletion, and/or insertion of one or more nucleotides.

The invention also provides a polynucleotide encoding the amylase, an expression vector comprising the polynucleotide, a transformed host cell comprising the vector, as well as a method of producing the amylase by cultivating the transformant. The invention further pro-

vides a dough composition comprising the amylase, a method of preparing a dough-based product by leavening and heating the dough, e.g. by baking.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

5 A source organism of the amylase of the invention is a fungal strain isolated from soil samples collected from Hainan Province, China, in 2002. The strain was at first classified as *Chaetomium* sp. and was later re-classified as *Valsaria rubricosa* belonging to Diaporthales, Ascomycetes, Ascomycota. It was found to harbor an amylase gene shown in SEQ NO: 1. The inventors have cloned the gene into a strain of *E. coli* and deposited it under the terms of the
10 Budapest Treaty on 16 December 2003 as DSM 16113 with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE.

The inventors also found an amylase gene in *Valsaria rubricosa* CBS 848.96. This strain is available on commercial terms from Centraalbureau voor Schimmelcultures, Uppsala-
15 laan 8, P.O. Box 85167, 3584 CT, The Netherlands. *Valsaria rubricosa* CBS 848.96 was found to harbor an amylase gene shown in SEQ NO: 18

Polypeptide with amylase activity

The polypeptide with amylase activity has an amino acid sequence which includes a catalytic core which may be the catalytic core sequence encoded by a DNA sequence in a
20 plasmid present in *E. coli* DSM 16113, the sequence shown in positions 1-439 of SEQ ID NO 2, or a sequence having at least 70 % identity to said sequences. Also, the catalytic core may be a sequence encoded by a nucleic acid sequence which hybridizes at 55 °C with the complementary strand of nucleotides 146-1462 of SEQ ID NO: 1.

Optionally, the amino acid sequence may further comprise both the catalytic domain
25 and a carbohydrate-binding module (CBM), or it may include the catalytic domain without a CBM. The CBM may be that present in the donor strain or an analogue thereof, or an extrinsic CBM may be substituted, or one or more additional CBMs may be inserted.

The polypeptide may have the sequence shown as SEQ ID NO: 19.

Carbohydrate-binding domain (CBM)

30 The carbohydrate-binding module (CBM) or carbohydrate-binding domain (CBD) is a polypeptide which binds preferentially to a poly- or oligosaccharide (carbohydrate), particularly in water-insoluble (e.g. crystalline) form. It may particularly be a starch-binding modules (SBM) or starch-binding domain (SBD). The one or more CBMs may optionally further comprise one

or more polypeptide amino acid sequence regions linking the CBM(s) with the catalytic module(s), a region of the latter type usually being denoted a "linker".

The invention provides a polypeptide with an amino acid sequence including a CBM with at least 70 % identity to amino acids 440-566 of SEQ ID NO: 2.

5 Heterologous carbohydrate-binding modules

The CBM *per se* typically consists of more than about 30 and less than about 250 amino acid residues. The CBM may be a "Carbohydrate-Binding Module of Family 20" or a CBM-20 module, typically a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1
10 by Joergensen et al (1997) in *Biotechnol. Lett.* 19:1027-1031, where the CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683.

The CBM (or SBM) may be derived from a starch degrading enzyme (amylolytic enzyme), such as a glucoamylase (EC 3.2.1.3), a cyclodextrin glucanotransferases or CGTase
15 (EC 2.4.1.19), an alpha-amylase (EC 3.2.1.1) or a maltogenic alpha-amylase (EC 3.2.1.133). The CBM may be derived from fungal, bacterial or plant sources, e.g. derived from *Aspergillus* sp., *Bacillus* sp., *Klebsiella* sp., or *Rhizopus* sp. The CBM may also be in the form of a non-hydrolytic polysaccharide-binding protein, e.g. found in algae, such as red alga *Porphyra purpurea*. The CBM may be located at the N or C terminus or at an internal position in a polypep-
20 tide (e.g. an enzyme).

Further examples of CBMs are described in PCT/US2004/020499, incorporated herein by reference.

Combination of amylases

A combination of two amylases may be added to dough to achieve anti-staling in a
25 product made from the dough.

The first amylase may be a thermostable amylase which includes a CBM, particularly a fungal amylase. It may be an alpha-amylase which retains more than 50% activity after 15 min incubation at 62°C (or 64°C or 66°C) in 50 mM sodium acetate, 1 mM CaCl₂, pH 5.7. It may have less than 50% activity at 71°C (or 69°C) under the same conditions. An example is
30 the *Valsaria* amylase described above.

The second amylase may be an exo-amylase. It may be capable of hydrolyzing starch by cleaving off linear maltooligosaccharides, e.g. maltose, maltotriose or maltotetraose, from the non-reducing ends of amylopectin. One example is maltogenic alpha-amylase (EC 3.2.1.133) such as Novamyl® or a variant thereof, e.g. having at least 90 % amino acid identity
35 to Novamyl as described in US 6162628, where the Novamyl sequence is shown as SEQ ID NO: 1.

The exo-amylase may hydrolyze amylose (e.g. wheat amylose or synthetic amylose) so that the average molecular weight of the amylose after 0.4-4 % hydrolysis (i.e. between 0.4-4 % hydrolysis of the total number of bonds) is more than 50 % (particularly more than 75 %) of the value before the hydrolysis. The hydrolysis can be conducted at the conditions described above, and the molecular weight distribution before and after the hydrolysis can be determined by HPLC. The test may be carried out as described in C. Christophersen et al., Starch 50 (1), 39-45 (1998).

Recombinant expression vector

The expression vector of the invention typically includes a selectable marker and control sequences encoding a promoter, a 5' untranslated leader and, a transcription terminator. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The polypeptide of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the amylase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism may particularly be a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, e.g. a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. graminearum* or *S. cerevisiae*.

Hybridization

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involve presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ³²P-dCTP-labeled (specific activity > 1 x 10⁹ cpm/µg) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more particularly at least 60°C, more particularly at least 65°C, even more particularly at least 70°C, especially at least 75°C. Molecules to which the oligonucleotide probe hybridizes under these conditions may be detected using a x-ray film.

Alignment and identity

The polypeptide and polynucleotide of the invention may have identities to the disclosed sequences of at least 80 %, particularly at least 85 % or at least 90 %, e.g. at least 95 %.

5 For purposes of the present invention, alignments of sequences and calculation of identity scores may be done using a Needleman-Wunsch alignment (i.e. global alignment), useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in
10 a gap is -2 for proteins and -4 for DNA. Alignment is from the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Dough

15 The dough of the invention generally comprises flour, particularly wheat flour. The dough may be fresh, frozen or par-baked. It may be a laminated dough.

The dough may also comprise other conventional dough ingredients, e.g.: proteins, such as milk powder and gluten; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or
20 ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate. The dough may comprise fat (triglyceride) such as granulated fat or shortening.

Additional enzyme

Optionally, one or more additional enzymes may be added to the dough together with
25 the amylase(s) described above. The additional enzyme may be a lipolytic enzyme (e.g. as described in WO 9953769) or a xylanase.

Dough-based product

The invention provides a method for preparing a dough-based product by leavening the dough and heating it, e.g. by baking or steaming. The dough may be leavened e.g. by add-
30 ing chemical leavening agents or yeast, usually *Saccharomyces cerevisiae* (baker's yeast). The product may be of a soft or a crisp character, either of a white, light or dark type. Examples are steamed or baked bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls.

Amylase Units (AmU)

Amylase activity was assayed by incubating with Phadebas amylase test tablets (product of Amersham Pharmacia) suspended in 50 mM sodium acetate + 1 mM CaCl₂ at pH 5.7 and determining OD at 650 nm. The Amylase Unit (AmU) was defined by taking the activity of the commercial product BAN 480L (product of Novozymes A/S) as 480 AmU.

EXAMPLES**Materials and methods**

RNeasy Mini Kit (Qiagen, Cat. #74904).

Taq DNA polymerase (Promega, Cat. # M166A)

10 pGEM-T Vector System I (Promega, Cat. # A3600)

Wizard Plus Minipreps DNA Purification System (Promega, Cat. # A7510)

5' Rapid Amplification of cDNA End System (Life Technologies, 5'RACE, Cat. # 18374-041),

3' Rapid Amplification of cDNA End System (Life Technologies, 3' RACE, Cat. # 1085805)

ElectroMAX DH10B Cells (Life Technologies, Cat. # 18290-015)

Example 1: Cultivation of fungal strain for cDNA preparation

A fungal strain of *Valsaria rubricosa* was grown on YG agar plate (4.5 cm diam) for 5 days under 37°C in the darkness and used for inoculating shake flask. The plates with fully grown cultures were stored at 4°C before use.

To obtain the mycelium for cDNA library construction, 4-6 agar plugs with fully grown fungal cultures on the YG agar plates were used to inoculate one shake flask with FG-4 (50 ml in 500 ml Erlenmeyer flask with 2 baffles: 30 g Soymeal, 15 g Maltose, 5 g Peptone, 1000 ml H₂O, 1% starch, 1 g olive oil (2 drops / flask); Autoclave at 121 °C for 30 min) and grown under 37°C, 160 rpm for 24 hours. The mycelium was harvested by centrifugation of the culture broth at 8000 rpm and 4°C for 30 minutes. Then mycelium was transferred into a clean plastic bag following by immediately freezing in liquid nitrogen and stored at -80°C before total RNA was isolated.

Probe designing:

30 Degenerate primers were designed based on alignment of already known amylase gene sequences: amyD1 (SEQ ID NO: 3) and amyD2R (SEQ ID NO: 4).

The N-terminal amino acid sequence of the purified amylase AM835F was determined as shown in SEQ ID NO: 13. This was used to design four degenerate primers: AM835n-s1

(SEQ ID NO: 5), AM835n-s2a (SEQ ID NO: 6), AM835n-s2b (SEQ ID NO: 7), AM835n-s2c (SEQ ID NO: 8).

Extraction of total RNA:

Total RNA was isolated from the frozen mycelium of a strain of *Valsaria rubricosa* by using RNeasy Mini Kit according to the manufacturer's instructions.

Gene cloning:

cDNA was synthesized using 3' RACE kit. The primary PCR was performed by using N-terminal based degenerate primers (primer AM835n-s2 is a mixture of AM835n-s2a, b and c) with AUAP provided by the 3'RACE kit:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
100 micro-M AM835n-s2a	1 micro-l
100 micro-M AM835n-s2b	1 micro-l
100 micro-M AM835n-s2c	1 micro-l
AUAP	1 micro-l
cDNA	2 micro-l
Taq DNA polymerase (5u/ micro-l)	1 micro-l
H ₂ O	34 micro-l

10 The PCR program was: 94°C for 3 min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1.5 min; final extension at 72°C for 10 min.

There was no specific amplification seen when the PCR product was visualized under UV but this product was used for second PCR with degenerate primers designed based on amylase homology. The 2nd PCR was performed by using amylase probes (amyD1 and amyD2R) and using the primary PCR as template:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
100 micro-M amyD1	1 micro-l
100 micro-M amy D2R	1 micro-l
1 st PCR	1 micro-l
Taq DNA polymerase (5u/ micro-l)	1 micro-l
H ₂ O	37 micro-l

The PCR program was: 94°C for 3min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1min; final extension at 72°C for 10min. A specific band was amplified at ~700bps and this was confirmed to be amylase by sequencing.

Based on the above obtained partial sequence, new primers were designed for 5' and 3' end cloning. For 5' end cloning, after cDNA was synthesized initiated with amy835as1 (SEQ ID NO: 9) by 5'RACE kit, PCR was performed with primer pairs amy835as1 and AAP (provided by the kit). Then nested PCR was performed with primer pair amy835as3 (SEQ ID NO: 14) and AUAP by using primary PCR (amy835as1-AAP) as template. A fragment of ~600bp was obtained and confirmed by sequencing. For 3' end cloning, PCR was performed by using primer pair of amy835f1 (SEQ ID NO: 15) and AUAP and cDNA as template. The nested PCR was performed by using primer pair amy835f2 (SEQ ID NO: 10) with AUAP and 1st PCR as template. A fragment at ~600 bps was amplified and again confirmed by sequencing.

10 Then based on the cloned 5' and 3' end sequences, the 5' and 3' end primers for full length cloning was designed and used for full length cloning of the amylase AM835. By using cDNA synthesized by 3' RACE kit as template and probes amy835s00 (SEQ ID NO: 11) and amy835as01 (SEQ ID NO: 12) as primers:

10X PCR buffer	5 micro-l
25mM MgCl ₂	3 micro-l
10mM dNTP	1 micro-l
10 micro-M amy835s00	1 micro-l
10 micro-M amy835as01	1 micro-l
cDNA	2 micro-l
Taq DNA polymerase (5 u/micro-l)	1 micro-l
H ₂ O	36 micro-l

15 PCR program was: 94°C for 3 min; 30 cycles of 94 °C for 30s, 50°C for 30s, 72°C for 1.5 min; final extension at 72°C for 10 min.

A specific fragment of ~2.0 kb was PCR-amplified. The fragment was cloned into pGEM-T vector (Promega) which has a 3'-T overhang and transformed into *E.coli* DH10B (ElectroMAX DH10B Cells, available from Life Technologies, Cat. # 18290-015) and further sequenced.

20 **Example 2: Production of amylase**

YG and FG-4 media were prepared as follows:

YG: Yeast-glucose agar

5.0 g Difco powdered yeast extract;	10.0 g glucose
20.0 g agar;	1000 ml tap water

25 Autoclave at 121°C for 15-20 min.

FG-4 Media 50 ml / flask:

30 g Soymeal,	15 g Maltose
---------------	--------------

5 g Peptone,

1000 ml H₂O

1 g olive oil (2 drops / flask)

50 ml in 500 ml Erlenmeyer flask with 2 baffles. Autoclave at 121 °C for 30 min.

A strain of the thermophilic fungus *Valsaria rubricosa* was grown on YG agar plate
5 (4.5 cm diam) for 3 days under 37°C in the darkness and used for inoculating shake flask. The
plates with fully grown cultures were stored at 4°C before use.

For enzyme production, 4-6 agar plugs with fully grown fungal cultures on the above
plates were used to inoculate one shake flask with FG-4 and grown under 37°C, 160 rpm for
72 hours, then harvested by centrifuged the culture broth at 8000 rpm and 4°C for 30 minutes.
10 The supernatant was collected and used for enzyme purification.

1000 ml supernatant was precipitated with ammonium sulfate (80% saturation) and
redissolved in 100 ml 25mM Tris-HCl buffer, pH7.0, then dialyzed against the same buffer and
filtered through a 0.45 mm filter, the final volume was 200 ml. The solution was applied to a 35
ml Source 15Q column (Pharmacia) equilibrated in 25 mM Tris-HCl buffer, pH7.0, and the pro-
15 teins was eluted with a linear NaCl gradient (0 –0.3M). Fractions from the column were ana-
lyzed for amylase activity on AZCL-amylose at pH 5.5. Fractions with amylase activity were
pooled. Then the pooled solution was ultrafiltrated, the concentrated solution was applied to a
180ml Superdex75 column equilibrated with 25 mM Tris-HCl, pH7.0, the proteins was eluted
with the same buffer. Amylase containing fractions were analyzed by SDS-PAGE and pure
20 fractions were pooled.

The purified amylase was used for characterization in the following example.

Example 3: Expression of an amylase from *Valsaria rubricosa* in *Aspergillus oryzae*

The DNA sequence of the *Valsaria rubricosa* amylase (SEQ ID NO.: 1) was used to
design primers for PCR amplification of the amylase encoding-gene from the clone described
25 in Example 1, with appropriate restriction sites added to the primer ends to facilitate sub-
cloning of the PCR product (primers AM835.1 and AM835.2, SEQ ID NO: 16 and 17). PCR
amplification was performed using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Fos-
ter City, CA, USA) following the manufacturer's instructions and using an annealing tempera-
ture of 55°C for the first 5 cycles and 65°C for an additional 25 cycles and an extension time of
30 2 minutes.

The PCR fragment was restricted with *Bam*HI and *Xho*I and cloned into the *Aspergil-*
lus expression vector pMStr57 using standard techniques. The expression vector pMStr57
contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to
the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has
35 sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergil-*
lus. Specifically, selection in *Aspergillus* is facilitated by the *amdS* gene of *Aspergillus nidu-*

lans, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (tpi) encoding-gene from *Aspergillus nidulans*, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The amylase-encoding gene of the resulting *Aspergillus* expression construct, pMStr91, was sequenced and the sequence agreed completely with that determined previously.

The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr91 using standard techniques (Christensen, T. et al., (1988), Biotechnology 6, 1419-1422). Transformants were cultured in YP+2%G medium shaken at 250 RPM at 30°C and expression of amylase was monitored by SDS-PAGE.

Medium YP+2%G

10g yeast extract
20g peptone
15 water to 1L
autoclave at 121°C, 20 minutes
add 100ml 20% sterile glucose solution

Example 4: Characterization of amylase

The molecular weight of the amylase prepared in a previous example was found to be around 66 kDa as seen on SDS-PAGE. The isoelectric point (pI) was found to be around pH 3.5, as determined by isoelectric focusing (IEF).

pH and temperature profiles were determined with AZCL-amylose (product of Megazyme) as substrate. At 50°C, the amylase was found to be active at pH 4-10 with an optimum around pH 5-7. At pH 5.5, the amylase was found to be active at 20-70°C with an optimum around 60°C. Thus, the *Valsaria rubricosa* amylase has a wider pH range and a higher temperature optimum than the fungal amylase from *Aspergillus oryzae*.

Stability of the amylase was determined by incubation at pH 5-7 and 60-80°C for 5-25 minutes. The results showed more than 90 % residual activity after 20 minutes at pH 6-7 and 60°C. At pH 5.0 and 60°C, the amylase was nearly completely inactivated in 15 minutes. At 70°C, the amylase was nearly completely inactivated at in 5-10 minutes at pH 6-7. It was found that at all conditions the *Valsaria rubricosa* amylase is more stable than the fungal amylase from *Aspergillus oryzae*.

The amylase showed no activity on the following substrates at pH 7.0: AZCL-galactomannan, AZCL-beta-glucan, AZCL-dextran, AZCL-xyloglucan, AZCL-potato galactan, AZCL-arabinan, AZCL-pullulan, AZCL-xylan, AZCL-he-cellulose and AZCL-casein.

Example 5: Effect of amylase on freshness of bread

Bread were baked according to the sponge & dough method.

Recipes

<u>Sponge</u>	<u>% on flour basis</u>
Soya oil	2,5
Sodium stearoyl lactylate (SSL)	0,38
Yeast	5
Wheat flour	60
Water	62
 <u>Dough</u>	 <u>% on flour basis</u>
Ascorbic acid	optimized for each flour
ADA	20 ppm
Salt	2
Syrup	7 (dry substance)
Water	optimized for each flour
Wheat flour	40
Calcium propionate	0.25
Enzymes	as indicated below

Sponge

- 5 Scaling of ingredients, addition of yeast, water, flour, SSL and oil into mixer bowl
 Mixing 90 rpm for 1 minutes, 150 rpm for 4 minutes
 The sponge was weighted, the temperature was measured and the sponge was placed in a bowl ~ fermentation 3 hours at 27 C, 86 % RH

Dough

- 10 Addition of ingredients and the sponge into the mixer bowl. The sponge and ingredients were mixed together 90 rpm for 9 minutes
 The temperature was measured, dough characteristics were evaluated, the dough was scaled into smaller pieces of 435 g each.
 The dough rests on the table for 10 minutes
 15 Doughs were sheeted and molded.
 Fermentation for 55 minutes at 42°C and 86% RH.
 Bread were baked at 200°C for 22 minutes
 Enzymes were dosed at 400 MANU/kg of Novamyl together with 0, 5 or 20 AmU/kg of the amylase of SEQ ID NO: 2 (prepared as in Example 1).

Bread were stored at room temperature until analysis.

Texture and water migration by NMR were measured on day 7, 14 and 21. A small sensory evaluation of softness and moistness was performed on day 21.

Results

- 5 Firmness of the loaves was measured as described in WO 9953769 The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Firmness after 7 days g	Firmness after 14 days g	Firmness after 21 days g
400	0	593	869	1103
400	5	505	814	1000
400	20	480	789	939

Elasticity of the loaves was measured as described in US 6162628. The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Elasticity after 7 days %	Elasticity after 14 days %	Elasticity after 21 days %
400	0	50.7	46.5	45.2
400	5	50.1	46.7	44.7
400	20	50.7	47.2	46.0

10

The data show that the amylase of the invention has a significant effect on firmness in combination with Novamyl, furthermore the elasticity seems to be comparable to or even better than that of Novamyl after 21 days of storage.

- 15 The mobility of free water was determined as described by P. L. Chen, Z. Long, R. Ruan and T. P. Labuza, Nuclear Magnetic Resonance Studies of water Mobility in Bread during Storage. Lebensmittel Wissenschaft und Technologie 30, 178-183 (1997). The results were as follows:

Novamyl dosage MANU/kg	Amylase of in- vention AmU/kg	Free water after 7 days Micro-sec	Free water after 14 days Micro-sec	Free water after 21 days Micro-sec
400	0	7498	6921	6198
400	5	7780	6856	6424
400	20	7945	7004	6618

The data show that the amylase of the invention increases the amount of free water. The amount of free water has been described in literature to correlate to moistness of bread crumb.

The ranking from the small sensory evaluation of softness and moistness on day 21
5 showed the following ranking (MANU/kg of Novamyl + AmU/kg of amylase of invention):

Moistest: 400 MANU + 20 AmU

Second: 400 MANU + 5 AmU

Lowest (least moist): 400 MANU

PCT

Original (for SUBMISSION)

(This sheet is not part of and does not count as a sheet of the international application)

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.50 (Build 0002.162)
0-2	International Application No.	
0-3	Applicant's or agent's file reference	10583.204-WO
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	2
1-2	line	10-12
1-3	Identification of deposit	
1-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroor- ganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	16 December 2003 (16.12.2003)
1-3-4	Accession Number	DSMZ 16113
1-5	Designated States for Which Indications are Made	all designations

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	
0-4-1	Authorized officer	

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	